Crimean-Congo Hemorrhagic Fever Virus in Humans and Livestock, Pakistan, 2015–2017

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We detected Crimean-Congo hemorrhagic fever virus infections in 4 provinces of Pakistan during 2017–2018. Overall, seroprevalence was 2.7% in humans and 36.2% in domestic livestock. Antibody prevalence in humans was highest in rural areas, where increased contact with animals is likely.

Crimean-Congo hemorrhagic fever (CCHF) is caused by CCHF virus (CCHFV), an emerging zoonotic virus belonging to the order Bunyavirales within the family *Nairoviridae*. The virus is maintained through a tick–vertebrate transmission cycle (*1*); the primary vectors are ticks from the genus *Hyalomma* (*2*,*3*). Wild and domestic mammals, including livestock species such as sheep, goats, and cattle, are amplifying hosts (*2*). CCHFV is listed as a high-priority zoonotic pathogen of humans in the World Health Organization Research and Development Blueprint (https://www.who.int/blueprint/priority-diseases) because of its potential to cause a public health emergency and the absence of specific treatment and vaccines.

Most human infections occur through the bite of infected ticks. Blood and other bodily fluids of infected animals represent an additional source for human infections. In humans, CCHF is manifested by fever, headache, vomiting, diarrhea, and muscular pain; bleeding diathesis with multiorgan dysfunction is seen in severe cases (*4*–*6*). CCHFV is endemic over a wide geographic area, spanning from western Asia to southern Europe and over most of Africa (*2*). Since the earliest identified CCHF case in 1976 (*7*), several outbreaks of CCHFV infection have been reported from Pakistan. Although Pakistan has the fourth highest number of human cases in Asia (*2*), no comprehensive surveillance study has been conducted to determine the disease prevalence in human and animal populations of Pakistan. Therefore, we determined the countrywide risk for CCHFV infection by detecting the virus and antibodies in livestock, ticks, and humans.

The Study

During 2017–2018, we tested 3,710 serum samples from 1,872 humans and 1,838 domestic animals (311 buffaloes, 480 camels, 183 cattle, 440 goats, and 424 sheep) for antibodies against CCHFV (Appendix, https://wwwnc.cdc.gov/EID/article/26/4/19-1154-App1.pdf). We also screened 98 blood plasma samples (24 from goats, 28 from buffalo, and 46 from cows) and 774 ticks (509 *Hyalomma* spp., 134 *Rhipicephalus* spp., 77 *Haemaphysalis* spp., and 54 *Rhipicephalus* [*Boophilus*] spp.), sampled from livestock in Punjab Province, for CCHFV antigen by commercial ELISA (VectoCrimea-CHF-antigen ELISA; Vector-Best, https://vector-best.ru).

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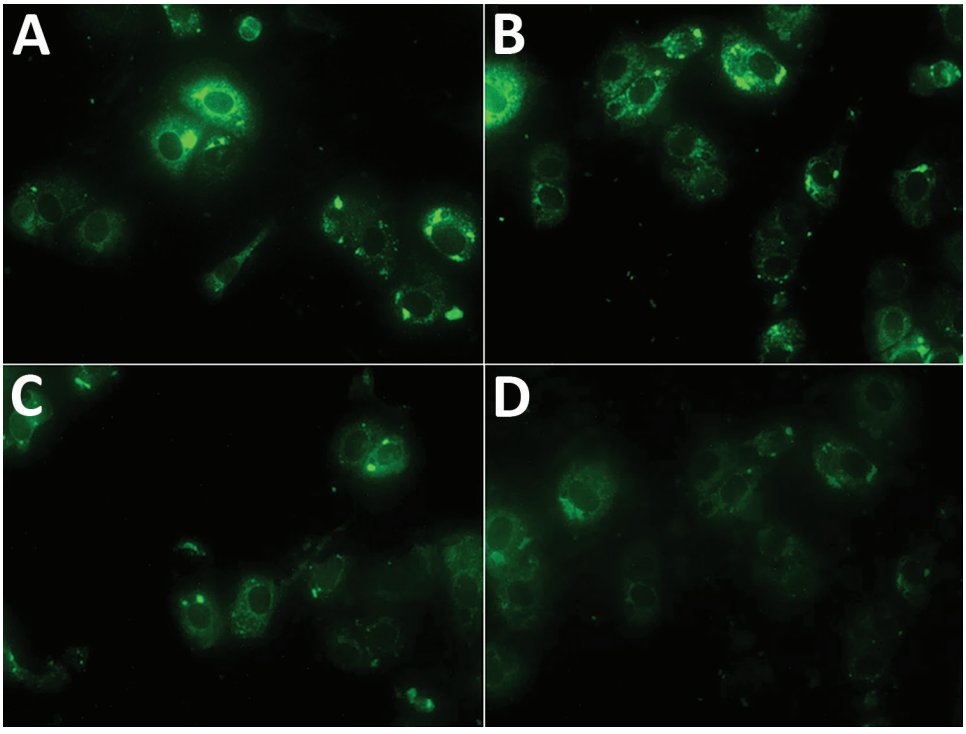

Figure 1. Indirect immunofluorescence assay results for Crimean-Congo hemorrhagic fever virus for 4 samples from humans that were positive by ELISA, Pakistan, 2016–2017. A, B) Samples at 1:100 dilution. C, D) Samples at 1:20 dilution. Original magnification ×100.

We found a total of 51 (2.7%) human samples to be positive for CCHF antibodies by using a 2-step approach, ELISA and confirmatory testing by immunofluorescence assay (Figure 1; Appendix). We observed significantly higher than average prevalence (p<0.01) among samples from Balochistan (5.7%, 95% CI 3.4%–9.3%); and the lowest prevalence among those from Sindh (1.1%, 95% CI 0.5%–2.3%). Samples from Balochistan were almost 6 times (odds ratio [OR] 5.6, CI 2.0–18.0) more likely to test positive than those from Sindh. Seroprevalence increased uniformly with age; we saw the highest level of CCHFV antibodies in persons ≥65 years of age (Table 1). Of the 51 positive samples, 28 (2.7%, 95% CI 1.8%–3.8%) were from female and 23 (2.8%, 95% CI 1.9%–4.2%) from male participants. We observed significantly higher (p<0.01) seroprevalence among livestock farmers (3.2%, 95% CI 2.4%–4.2%) compared with the general population (0.6%, 95% CI 0.1%–2.3%).

Of the 1,838 animals, 666 (36.2%) were positive for CCHF by a commercial ELISA (ID Vet, https://www.id-vet.com). The prevalence of CCHFV antibodies was significantly higher (p<0.01) among camels (56.7%, 95% CI 52.1%–61.2%) than among cattle (44.3%, 95%

Table 1. Univariate analyses of 1,872 human samples positive for Crimean-Congo hemorrhagic fever virus by ELISA, Pakistan, 2017–2018

Category	No. positive/no. tested	Prevalence, % (95% CI)	Odds ratio (95% CI)	p value
Province				<0.001
Punjab	25/930	2.7 (1.8–4.0)	2.6 (1.0–7.7)	
Khyber Pakhtunkhwa	6/128	4.7 (2.1–10.0)	4.6 (1.2–17.5)	
Balochistan	14/247	5.7 (3.4–9.3)	5.6 (2.0–18.0)	
Sindh	6/567	1.1 (0.5–2.3)	1.0	
Age, y				0.451
15–24	7/438	1.6 (0.8–3.3)	1.0	
25–34	19/730	2.6 (1.7–4.0)	1.6 (0.7–4.7)	
35–44	12/388	3.1 (1.8–5.4)	2.0 (0.7–6.0)	
45–54	9/226	4.0 (2.1–7.5)	2.6 (0.8–8.2)	
55–64	3/70	4.3 (1.4–12.5)	2.8 (0.5–12.4)	
≥65	1/20	5.0 (0.7–28.2)	3.2 (0.1–27.3)	
Sex				0.832
F	28/1,055	2.7 (1.8–3.8)	1.0	
M	23/817	2.8 (1.9–4.2)	1.1 (0.6–1.9)	
Occupation				0.006
Livestock farmer	49/1,523	3.2 (2.4–4.2)	5.8 (1.5–49.2)	
General population	2/349	0.6 (0.1–2.3)	1.0	

Table 2. Univariate analyses of 1,838 livestock samples positive for Crimean-Congo hemorrhagic fever virus by ELISA, Pakistan, 2017–2018

Category	No. positive/no. tested	Prevalence, % (95% CI)	Odds ratio (95% CI)	p value
Species				<0.001
Camel	272/480	56.7 (52.1–61.2)	5.6 (4.2–7.6)	
Cattle	81/183	44.3 (36.9–51.8)	3.4 (2.3–5.0)	
Sheep	138/424	32.6 (28.1–37.2)	2.1 (1.5–2.8)	
Buffalo	92/311	29.6 (24.6–35.0)	1.8 (1.3–2.5)	
Goat	83/440	18.9 (15.3–22.8)	1.0	
Province				<0.001
Balochistan	213/359	59.3 (54.1–64.5)	7.6 (5.4–10.6)	
Khyber Pakhtunkhwa	230/439	52.4 (47.6–57.1)	5.7 (4.1–7.9)	
Punjab	159/644	24.7 (21.4–28.2)	1.7 (1.2–2.40)	
Sindh	64/396	16.2 (12.7–20.2)	1.0	
Sex				0.377
F	552/1,504	36.7 (34.3–39.2)	1.1 (0.9–1.4)	
M	114/334	34.1 (29.1–39.5)	1.0	
Age, y				<0.001
≤5	332/1,121	29.6 (27–32.4)	1.0	
>5	334/717	46.6 (42.9–50.3)	2.1 (1.7–2.5)	

CI 36.9%–51.8%), sheep (32.6%, 95% CI 28.1%–37.2%), buffalo (29.6%, 95% CI 24.6%–35%), and goats (18.9%, 95% CI 15.3%–22.8%) (Appendix Tables 1–5). Camels were almost 6 times (OR 5.6) more likely to be positive than other species. As we found for humans, we found significantly higher (p<0.01) seroprevalence of CCHFV antibodies among animals from Balochistan (59.3%, 95% CI 54.1%–64.5%) than among animals from the other regions tested (Table 2).

We built a binary logistic regression model to evaluate possible risk factors for CCHFV seropositivity in animals and humans. The final model (Appendix Table 6) at the animal level indicated that the animals with highest risk for being antibody positive are livestock from Balochistan (OR 12.1, 95% CI 7.7–19.1), buffalo (OR 4.4, 95% CI 2.8–6.8), and animals >5 years of age (95% OR 1.3, CI 1.0–1.7). However, the NR^2 value of 0.277 and the Hosmer-Lemeshow goodness-of-fit test (χ^2 22.005; p = 0.003) indicated that this is a poor model for predicting CCHFV exposure in the sampled livestock population.

In humans, we found the chance of exposure to CCHFV was highest for populations from Balochistan (OR 6.6, 95% CI 2.5–17.5) (Appendix Table 7) and in persons belonging to the herdsman profession (OR 7.3, CI 1.7–30.2). The values of NR^2 (0.070) and Hosmer-Lemeshow goodness-of-fit test (χ^2 1.490; p = 0.684) indicated that our model is a reasonable model for predicting past exposure to CCHFV in the tested human population.

Four plasma samples from buffalo and 4 *Rhipicephalus* tick samples tested positive for CCHFV antigen by ELISA. Of these 8 positive samples, we confirmed 1 tick (T61) and 3 buffalo samples (15B, 16B, and 17B) through partial amplification and sequencing of the small (S) segment (260 bp). The 4 partial sequences of CCHFV S segments (GenBank accession nos. MN135938–MN135941) were 97%–95% identical to virus sequences found in Afghanistan (accession no. JX908640.1), Iran (accession no. KX096702.1), and Oman (accession no. KY362516.1) and clustered together with genotype IV (Asia) (Figure 2). We obtained full-length sequences of the CCHFV S, medium (M), and large (L) segments (accession nos. MN135942–MN135944) from the tick sample by sequencing on a HiSeq3000 (Illumina, https://www.illumina.com). Phylogenetic trees for the S, M, and L segments showed that the T61 strain clustered with genotype IV (Asia) (Appendix Figures 1–3).

Conclusions

This countrywide study of CCHFV in Pakistan strongly suggests virus circulation in specific geographic regions and suggests CCHFV foci and a potential source of human infections. Detection of the antibodies in domestic livestock species (including sheep, goats, cattle, buffalo, and camels) indicates a potential role of these animals in human infections. Demonstration of the virus in animal blood plasma and tick samples by reverse transcription PCR provides strong evidence of active circulation of CCHFV in Pakistan. Furthermore, genetic characterization of the virus reconfirms the circulation of genotype IV in Pakistan (*8*). Of interest, we found no *Hyalomma* tick positive for CCHFV; CCHFV has been reported from *Rhipicephalus* ticks from Iran and clustered together with strains from Pakistan and Iran, indicating that *Rhipicephalus* ticks have been naturally infected with closely related virus in the region (*9*). Our study further confirms the role of *Rhipicephalus* ticks in CCHFV circulation in the region. We observed higher prevalence of CCHFV antibodies in camels than in animals

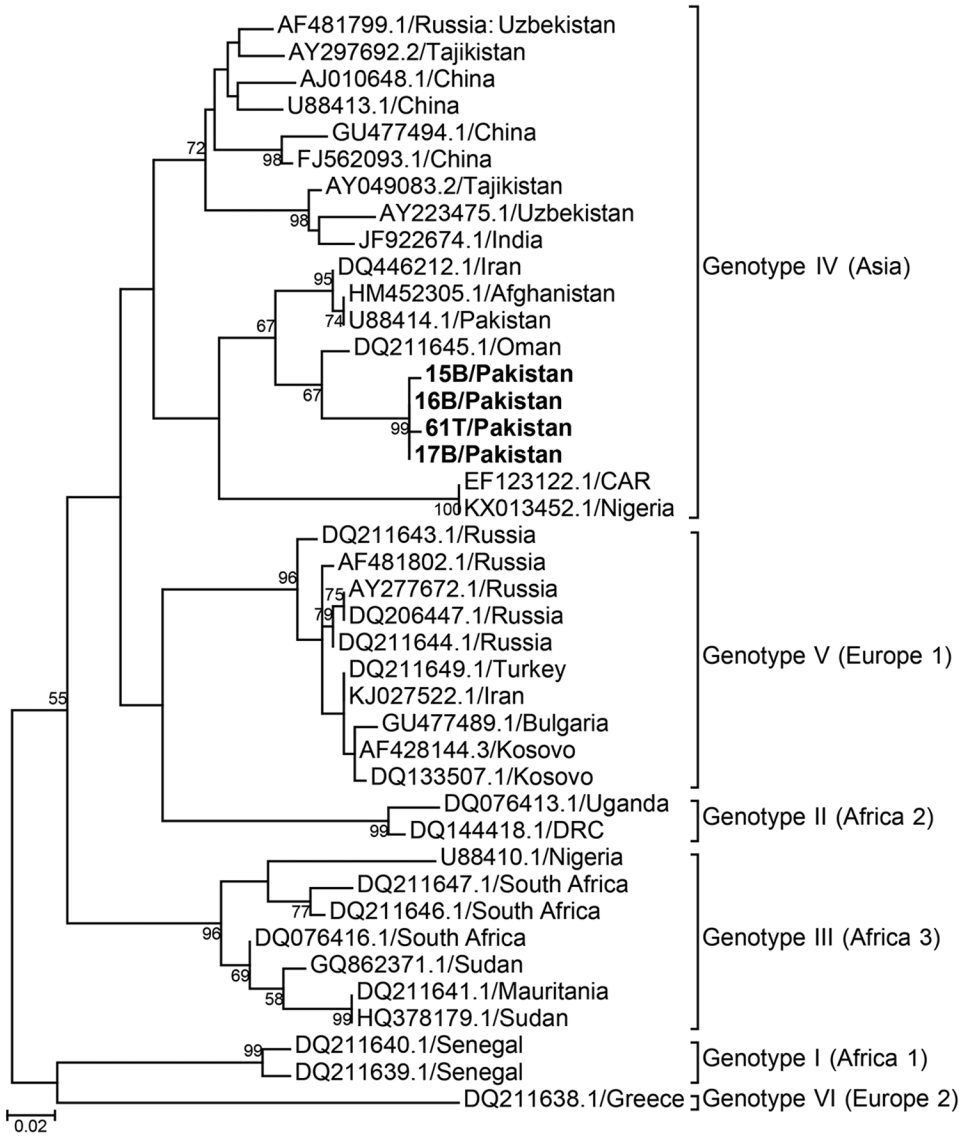

Figure 2. Phylogeny of Crimean-Congo hemorrhagic fever virus, Pakistan, 2016–2017 (bold text), and reference viruses, based on partial small gene sequences. Numbers at branch nodes indicate bootstrap support values. GenBank accession numbers are provided for reference sequences. Scale bar indicates nucleotide substitutions per site.

of other species, indicating the importance of camels in CCHFV ecology in Pakistan.

A high proportion of seropositive humans from Balochistan and Khyber Pakhtunkhwa with a history of exposure to animals is in concordance with earlier reports of CCHF in humans from these areas. The rural economy of Balochistan and Khyber Pakhtunkhwa is based on livestock production, and the increased contact with animals may explain the higher antibody prevalence in humans from these areas. Furthermore, the prevalence of antibodies was significantly higher among herdsmen than among the general population.

In summary, our results indicate the ongoing circulation of CCHFV among animals and humans in some regions of Pakistan. Longitudinal surveys to identify and define the genomic diversity of CCHFV in Pakistan and investigations to explore the exact role of camels in the ecology of this virus would help clarify the risk to the general population and occupational hazards for livestock farmers and veterinarians.

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Appendix

Human Samples

Human samples used in this study were part of the samples collected randomly during a countrywide arbovirus surveillance study conducted under the International Cooperation on Key Technologies of Biosafety along the China-Pakistan economic corridor. Due to limited availability of resources, troublesome geopolitical status, and difficulty in convincing farmers to provide samples, convenience sampling was performed. Because seroprevalence of the disease under study was unknown in these areas of Pakistan, the sample size was calculated by considering the expected prevalence to be 50% with confidence limits of 95% and desired absolute precision of 5% to take maximum numbers of samples (*1*). We collected a total of 1,872 human serum samples during 2017–2018. We also recorded information on individual factors such as demographics, animal ownership, and education status. In total, we collected 930 (49.68%) samples from Punjab, 567 (30.29%) from Sindh, 247 (13.19%) from Balochistan, and 128 (6.84%) from Khyber Pakhtunkhwa. Of 1,872 samples, 1,055 (56.36%) were from female and 817 (43.64%) from male participants. Humans sampled in this study were divided into 6 age groups: 15–24 years (n = 438), 25–34 years (n = 730), 35–44 years (n = 388), 45–54 years (n = 226), 55–64 years (n = 70) and ≥65 years (n = 20). Mean age of the sampled population was 33.36 years.

A clinician or phlebotomist collected 4 mL of blood from consenting study participants from the peripheral vein into the gel-clot activator containing vacutainer (Improvacuter, Germany). The samples were shipped to the University of Agriculture, Faisalabad, Pakistan, while maintaining the cold chain. Samples were then centrifuged at 5,000 rpm for 12 minutes; then serum was harvested and aliquoted in cryovials (Imec,China) and preserved at –40°C until subjected to further experimentation.

Animal and Tick Samples

For animal sampling, we selected 14 districts from Punjab, 3 from Khyber Pakhtunkhwa, 7 from Balochistan, and 5 from Sindh. We chose sampling sites on the basis of animal populations, ease of sampling, and the presence of veterinary clinics. We sampled a total of 311 buffaloes, 480 camels, 183 cattle, 440 goats, and 424 sheep; from each animal, we drew ≈4 mL blood aseptically directly into the gel-clot activator containing vacutainer (Improvacuter). We centrifuged samples, and then harvested serum samples and stored them at −40°C. The animal sampling period spanned 30 months during 2015–2018. In addition, Punjab Livestock and Dairy Development Department provided 98 plasma samples (24 from goats, 28 from buffaloes, and 46 from cows) from the household livestock of the CCHF-suspected human case-patients.

We collected a total of 509 *Hyalomma* spp., 134 *Rhipicephalus* spp., 77 *Haemaphysalis* spp., and 54 *Rhipicephalus* spp. ticks from livestock from Punjab province of Pakistan. The ticks were collected at the time of field sampling. Animals randomly selected for serum collection were also carefully examined for the presence of ticks; if infested, we collected the ticks by removing them with forceps from the animals. We stored ticks at 4°C in the field and sent them to University of Agriculture Faisalabad, Pakistan, where they were stored at −40°C.

Serologic Testing

For human serum samples, we used a 2-step approach. In the first step, we screened all human serum samples using a commercial ELISA (Vector-Best, https://vector-best.ru) according to the manufacturer's instructions. In the second step, all ELISA-positive serum samples were confirmed by indirect immunofluorescence assay. The cells were fixed with phosphate-buffered saline (PBS, pH = 7.5) containing 4% paraformaldehyde, permeablised with 0.2% TritonX-100, and blocked with 5% bovine serum albumin at 37°C for 1 hour. We added serum samples diluted in 1% bovine serum albumin and incubated plates overnight at 4°C. We washed the culture plates and added FITC-tagged anti-human IgG at a dilution of 1:1,000 (Sigma, USA). We incubated plates for 1 hour, then washed them and added 100 μL of 1× PBS solution. We observed fluorescence using a fluorescence microscope and acquired images. We first tested serum samples at a dilution of 1:100; negative samples were further tested at a dilution of 1:20. We used anti-CCHFV NP monoclonal antibody (43E5) as the positive control.

For animal serum samples, we conducted screening of anti-CCHFV IgGs by ELISA (ID Vet, https://www.id-vet.com). This double antigen multi-species ELISA can detect IgG antibodies against NP protein of CCHFV in caprine, ovine, bovine, and other susceptible species' serum samples.

We used animal plasma samples and ticks to screen CCHFV antigen by VectoCrimea-CHF-antigen (Vector-Best, https://vector-best.ru) ELISA kit according to the manufacturer's instructions.

Reverse Transcription PCR (RT-PCR)

We extracted total RNA from CCHFV antigen–positive samples using TRIzol reagent (Invitrogen, https://www.thermofisher.com) and performed RT-PCR as previously described (*2*). We visualized the PCR product on 0.8%–1% agarose gel after electrophoresis, then performed Sanger sequencing and edited sequences using Geneious R11. We performed BLAST searches using the nonredundant database (https://blast.ncbi.nlm.nih.gov/Blast.cgi). We performed multiple sequence alignment using MAFFT alignment plugin in Geneious R11 (https://www.geneious.com). We performed phylogenetic analysis in MEGA version 7.0 software (https://www.megasoftware.net).

Whole-Genome Sequencing and Analysis

We prepared libraries for next-generation sequencing using Truseq mRNA kit (TruSeq Stranded mRNA Library Prep Kit, Cat # RS-122–2101; Illumina) following the manufacturer's instructions. We performed sequencing on a HiSeq 3000 sequencer (). We analyzed the data in Metavisitor (a suite of galaxy tools) as described previously (*3*). We then used the BLAST guided scaffold to reference align the reads in Geneious R11. We set up RT-PCR reaction to fill the gap in L segment by using 3508 F(CCHF-L): GGCCAGCTTATCACTCATGGA and 3974 R(CCHF-L): CATTCTGCTGCCACCTCCTT primers.

Statistical Analysis

We conducted statistical analysis using R version 3.5.1 (https://www.r-project.org). We investigated positive samples by χ^2 test for the association of important host (location, sex, and

age) risk factors with the occurrence of infection in animals and human populations of Pakistan. We used the analysis packages Epicalc version 2.15.1.0 and DescTools version 0.99.25. For all analyses, $p<0.05$ was considered significant.

We built a binary logistic regression model to evaluate the risk factors for the seroprevalence of CCHFV in animals and humans. We entered all the variables with $p<0.20$ at univariable analysis into the model. We performed backward stepwise removal of the variables with $p>0.05$ until only significant ($p<0.05$) variables remained in the model. We assessed the fit of the final model by values of Nagelkerke R Square (NR^2) and Hosmer and Lemeshow (HL) goodness-of-fit test (*4*).

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Appendix Table 1. ELISA-based prevalence of Crimean-Congo hemorrhagic fever virus in camels, Pakistan

Variable	Category	No. positive/no. tested	Prevalence, % (95% CI)
Province	Punjab	59/184	32.1 (25.7–39.2)
	Khyber Pakhtunkhwa	88/165	53.3 (45.7–60.8)
	Balochistan	125/131	95.4 (90.2–97.9)
Age	<3 y	11/54	20.4 (11.7–33.2)
	3–10 y	221/356	62.1 (56.9–67.0)
	>10 y	40/70	57.1 (45.4–68.2)
Sex	Female	193/281	68.7 (63.0–73.8)
	Male	79/199	39.7 (33.1–46.7)

Appendix Table 2. ELISA-based prevalence of Crimean-Congo hemorrhagic fever virus in cattle, Pakistan

Variable	Category	No. positive/no. tested	Prevalence, % (95% CI)
Province	Punjab	31/117	26.5 (19.3–35.2)
	Balochistan	43/44	97.7 (85.6–99.7)
	Sindh	7/22	31.8 (16.0–53.4)
Age	<3 y	3/17	17.7 (5.8–42.7)
	3–10 y	75/149	50.3 (42.4–58.3)
	>10 y	3/17	17.7 (5.8–42.7)
Sex	Female	77/174	44.3 (37.1–51.7)
	Male	4/9	44.4 (17.7–74.9)

Appendix Table 3. ELISA-based prevalence of Crimean-Congo hemorrhagic fever virus in sheep, Pakistan

Variable	Category	No. positive/no. tested	Prevalence, % (95% CI)
Province	Punjab	11/100	11.0 (6.2–18.8)
	Khyber Pakhtunkhwa	85/182	46.7 (39.6–54.0)
	Balochistan	42/136	30.9 (23.7–39.1)
	Sindh	0/6	0 (0)
Age	<8 mo	13/32	40.6 (25.3–58.1)
	8 mo–4 y	81/261	33.6 (26.0–42.1)
	>4 y	44/131	31.0 (25.7–76.9)
Sex	Female	123/382	32.2 (27.7–37.1)
	Male	15/42	35.7 (22.8–51.1)

Appendix Table 4. ELISA-based prevalence of Crimean-Congo hemorrhagic fever virus in buffaloes, Pakistan

Variable	Category	No. positive/no. tested	Prevalence, % (95% CI)
Province	Punjab	49/123	39.8 (31.6–48.7)
	Sindh	43/188	22.9 (17.4–29.4)
Age	<3 y	8/34	23.5 (12.2–40.5)
	3–10 y	68/243	28.0 (22.7–34.0)
	>10 y	16/34	47.1 (31.2–63.5)
Sex	Female	87/299	29.1 (24.2–34.5)
	Male	5/12	41.7 (18.5–69.2)

Appendix Table 5. ELISA-based prevalence of Crimean-Congo hemorrhagic fever virus in goats, Pakistan

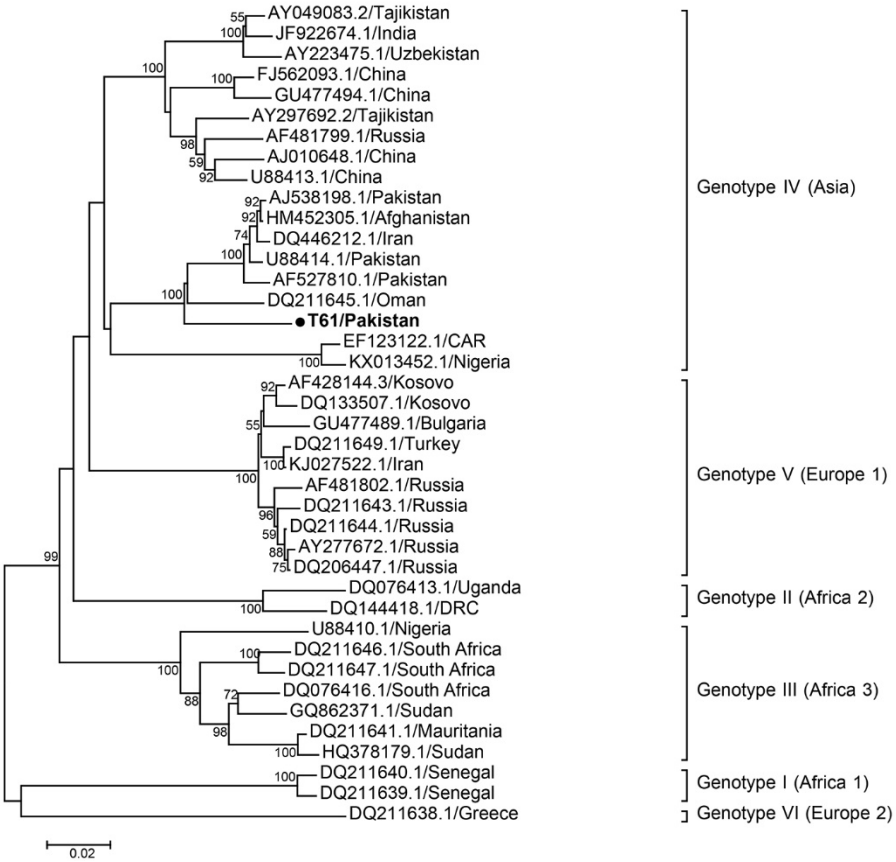
Variable	Category	No. positive/no. tested	Prevalence, % (95% CI)
Province	Punjab	9/120	7.5 (4.0–13.8)
	Khyber Pakhtunkhwa	57/92	62.0 (51.7–71.3)
	Balochistan	3/48	6.3 (2.0–17.7)
	Sindh	14/180	7.8 (4.7–12.7)
Age	<1 y	4/42	9.5 (3.6–22.8)
	1–4 y	70/322	21.7 (17.6–26.6)
	>4 y	9/76	11.8 (6.3–21.2)
Sex	Female	72/368	19.6 (15.8–23.9)
	Male	11/72	15.3 (8.7–25.5)

Appendix Table 6. Final binary logistic regression model to predict the Crimean-Congo hemorrhagic fever virus exposure in the sampled livestock population

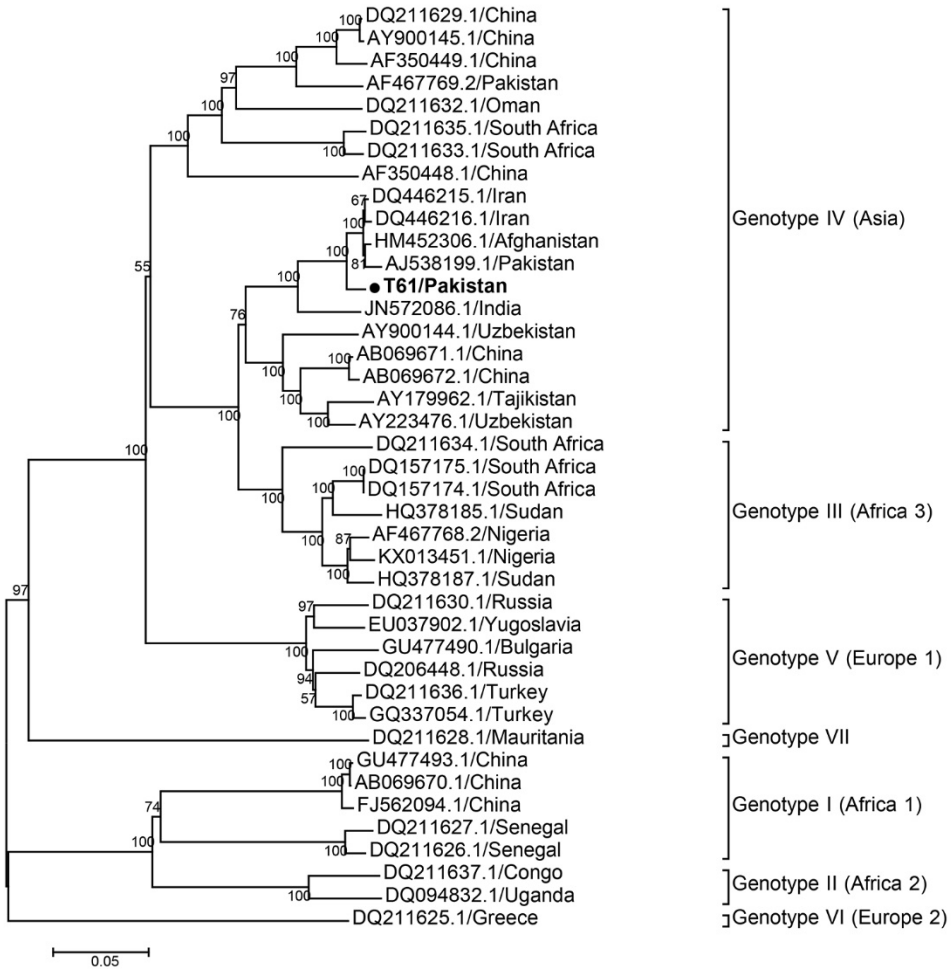
Variable	Category	Reference	Odds ratio (95% CI)	p value
Province	Balochistan	Sindh	12.1 (7.7–19.1)	<0.001
	Khyber Pakhtunkhwa	Sindh	10.8 (6.9–16.9)	
	Punjab	Sindh	1.7 (1.1–2.4)	
Species	Camel	Goat	3.3 (2.3–4.7)	<0.001
	Cattle	Goat	4.3 (2.7–6.8)	
	Sheep	Goat	0.9 (0.6–1.3)	
	Buffalo	Goat	4.4 (2.8–6.8)	
Age	>5 y	≤5 y	1.3 (1.0–1.7)	0.048

Appendix Table 7. Final binary logistic regression model to predict the Crimean-Congo hemorrhagic fever virus exposure in the sampled human population

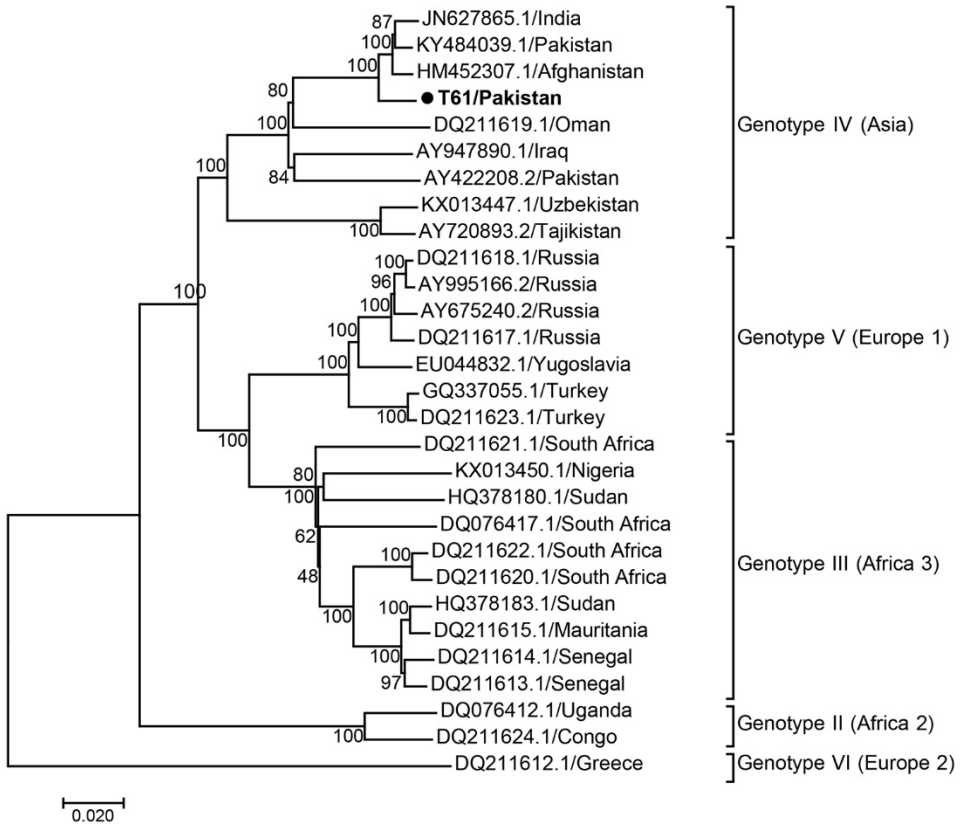
Variable	Category	Reference	Odds Ratio (95% CI)	p value
Province	Balochistan	Sindh	6.6 (2.5–17.5)	0.001
	Khyber Pakhtunkhwa	Sindh	5.7 (1.8–18.1)	
	Punjab	Sindh	2.7 (1.1–6.5)	
Profession	Herdsman	Others	7.3 (1.7–30.2)	0.006



Appendix Figure 1. Phylogenetic analysis of Crimean-Congo hemorrhagic fever virus S segment from Pakistan, compared with reference sequences. Dot and bold text indicate strain detected in this study. Numbers at branch nodes indicate bootstrap support values. The scale bar indicates nucleotide substitutions per site. …



Appendix Figure 2. Phylogenetic analysis of Crimean-Congo hemorrhagic fever virus M segment from Pakistan. Dot and bold text indicate strain detected in this study Numbers at branch nodes indicate bootstrap support values The scale bar indicates nucleotide substitutions per site. …



Appendix Figure 3. Phylogenetic analysis of Crimean-Congo hemorrhagic fever virus L segment from Pakistan, compared with reference sequences. Dot and bold text indicate strain detected in this study. Numbers at branch nodes indicate bootstrap support values. The scale bar indicates nucleotide substitutions per site